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SHERLOCK – A PLATFORM FOR NUCLEIC ACID DETECTION

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Abstract: *New analytic strategies based on recent technological achievements have a big impact on modern microbiology diagnostics. The genetic information stored in nucleic acids became the target of many molecular methods which are used to detect and identify different microorganisms. Awareness of the variety of pathogens that can manifest in a number of both local and systematic ways is the main concern in maintaining global health. So, the precise, rapid and reliable determination between causative agents enables an adequate response. The discovery and understanding of functioning mechanisms of Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) and their associated proteins (Cas) presented an enormous opportunity for developing several technologies that were and will be incorporated into clinical usage. Two of the Cas proteins, Cas12 and Cas13, nowadays are the main enzymes that tend to be combined with methods of nucleic acid pre-amplification in order to achieve sequence-specific recognition. Specific High Sensitivity Enzymatic Reporter UnLOCKing (SHERLOCK) is the prototype of the above mentioned union. Multiplexed, ultra-sensitive and highly specific detection of RNA or DNA from a particular clinical sample can be carried out with the SHERLOCK platform in a short period of time. Further research along with different implementations of this method will ensure continued improvement of analytic pathways that are an essential part of the microbial diagnostics. This paper explains main working principles of the SHERLOCK platform and point out the possibilities of its applications in clinical diagnostics.*

Key words: *SHERLOCK, nucleic acids, detection*

Introduction

The rapid, accurate and precise detection of an agent that caused a certain pathological condition has an important role in modern clinical diagnostics. Distinguishing between different microorganisms, especially those of the same genus, nowadays is quite simplified by targeting their nucleic acids in order to provide species-specific recognition. Several methods and techniques are in use but the major focus of many recent studies are those that combine amplification of the nucleic acid with sequence-specific detection by CRISPR/Cas system. Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) associated with Cas proteins provide highly specific recognition and cleavage of a certain region in the targeted genome. The cleavage with these "molecular scissors" shows great potential for developing new and upgrading the already implemented detection methods [1]. Certain Cas orthologues have the ability to maintain cleavage activity regardless of

the type of the nucleic acid present in the microbial cell. Class 2 Type V and type VI Cas proteins (Cas12 and Cas13, respectively) have different preferences but both can be applied, especially on a previously prepared (pre-amplified) sample. The amplification of the nucleic acid increases the possibility of detecting a specific sequence which will determine the type/species of the pathogen of interest. The combination of above mentioned methods is presented by Gootenberg et al. [2] as the Specific High Sensitivity Enzymatic Reporter UnLOCKing (SHERLOCK) platform [2]. The main principles of function along with opportunities of implementation of the SHERLOCK platform will be presented and discussed below.

CRISPR/Cas system

Analyzing the genome of *Escherichia coli*, in 1987 resulted in the discovery of a specific palindromic repetitive nucleotide array that was later labeled as CRISPR. After years of studying and researching it was established that this array, along with Cas proteins, has an important role in the immune response of the prokaryotic (bacterial and archaeal) cell carrying it. The defense against bacteriophages and foreign plasmids is based on "memory" that has been assembled in the CRISPR locus. Similar to the adaptive immune response of eukaryotic cells, CRISPR/Cas systems provide effective protection of the host genome when it is exposed to a foreign nucleic acid or mobile genetic elements. Trough stages of adaptation, expression and interference the acquisition of new spacers is made so the host cell manages to gain memory of a certain agent. The effector molecule that carries the main function (recognition and cleavage) of the CRISPR/Cas system is a complex of crRNA and a corresponding Cas nuclease (depending of the class and type of the system). The ability of this system to achieve sequence-specific recognition followed by uni- or collateral disintegration of the targeted nucleic acid is utilized for developing several diagnostic and therapeutic tools. [3]. The CRISPR/Cas system, recently widely analyzed for its application in molecular diagnostics, gene arrangement and gene therapy, is the type VI with its Cas13 effector protein [4].

Cas13 protein

RNA-based microorganisms (such as viruses) can be problematic to detect and identify because the majority of molecular techniques stream to DNA so the final results can give a false negative readout. This obstacle is successfully resolved with the development of the Cas13 nuclease-based system which utilizes the main characteristic of the mentioned protein - its preference to cleave single stranded RNA (ssRNA) molecules [5]. Even though Cas 13, as the effector of the type VI CRISPR/Cas system, is classified into class 2, it doesn´t share many similarities with the other protein in its class. The prior mentioned nuclease belongs to a family of ribonucleases that are guided by another RNA molecule (guide RNA, gRNA) and it is capable of generating multiple cleavage effects in the same targeted RNA with single-base specificity [6]. In order to selectively target a strictly specific sequence, Cas13 undergoes conformational change [7]. Another feature of this protein are its independence of PAM molecules (protospacer adjacent motif) and the ability to cause collateral cleavage in a specific region so that implicate even bigger usage in analyses

dedicated to pathogen detection [6]. Two regions of the Cas13 nuclease are NUC (nuclease domain) and REC (crRNA recognition domain), lobes that provide nuclease activity which results in collateral cleavage of the targeted RNA. Each of the lobes is composed of other domains with separate functions. An N-terminal domain (NTD) and a domain called Helical-1 together make the REC lobe, while the NUC lobe is consisted of two HEPN (higher eukaryotes and prokaryotes nucleotide-binding) domains joined with a linker and a helix-2 domain [8]. The orthologues of Cas13 protein include Cas13a, Cas13b, Cas13c, and Cas13d ribonucleases of which all four have the same cleavage ability with slight difference in constitution and size [9]. The disruption of the target and non-target RNA serves the severe opportunities of application in molecular diagnostics [8].

Nucleic acid amplification

Natural, raw samples are very complex so the differentiation and identification of foreign nucleic acids becomes quite challenging. In order to generate an adequate concentration of the target sequence, the freshly collected samples undergo considerable preparation stages to finally get to the system of nucleic acid amplification, which will increase the sensitivity of following detection by multiplying the desired sequence. The "gold standard" amplification method is polymerized chain reaction (PCR), which is capable to exponentially multiply a desired sequence starting from small amount of it (as few as 1 to 10 copies) [10]. Besides more than a few techniques with the same purpose, the focus here will be on the one that is commonly used as a compound of the SHERLOCK platform - recombinase polymerise amplification (RPA). RPA is an isothermal amplification process that performs on lower temperatures (37°C-42°C) in the aim to create an optimal environment for the activity of two effector enzymes (a recombinase and a DNA polymerase) [10]. This method is competent for amplifying either RNA or DNA molecules [11]. Due to its short operating time (20-40 minutes) it is deemed as one of the fastest methods for nucleic acid amplification. The amplicons are generated during the cyclic repetition of the recombinase-primer complex-based DNA scanning for locating homologous sequences, then followed by the forming of the D-loop structure and lastly the integration of dNTPs mediated by DNA polymerase [10]. Additionally, improvements of the RPA may add to its sensitivity and the ability to detect different molecules. An example of this is the development of reverse-transcription recombinase polymerized amplification (RT-RPA). The previously mentioned method also can be adapted in the SHERLOCK analytic flow.

SHERLOCK function principles

The SHERLOCK platform protocol includes nucleic acid amplification, T7 transcription and CRISPR/Cas13 collateral cleavage. The ability to spot single base differences at the attomolar level enables its application in a variety of analytic purposes including genotyping, pathogen detection and identification of free cancerous DNA [8]. The commonly used Cas13 protein is one derived from *Leptotrichia wandei* (LwCas13), non-motile facultative anaerobic bacteria that are a usual constituent of the oral microbiome. Usually, nucleic acid amplification is

maintained by the RPA or RT-RPA methods in aim to gain more copies of the target sequence. The second step includes T7 transcription which, based on the pre-amplified sample-derived nucleic acid, results in gaining numerous RNA amplicons. Obtained amplicons subsequently undergo specific collateral cleavage by the Cas13 protein and the resulting signal is generated as a fluorescence readout [7]. This is the consequence of the cleavage-based separation between a fluorophore and a quencher which are joined by a short RNA oligomer in the ssRNA reporter molecules [12]. The received fluorescence signal indicates the presence of the desired sequence in the original sample. From the abovementioned it can be concluded that the SHERLOCK platform is a triple signal system that, due to its high sensitivity, specificity and efficacy, provided by isothermal amplification and the CRISPR/Cas13 effect, has a huge potential for rapid quantitative detection of both previously known and emergent pathogens [8].

The variations of SHERLOCK platform

The association of different methods led to establishing a versatile tool that sums up the advantages of its constituents. When paired with specific sample preparation methods, or the addition of certain molecules to the reaction, the detection abilities of the already complex SHERLOCK platform may be increased. SHERLOCKv2 presents an extended version of the subject platform and it is known for four–point achievements that involve quadruple detection trough the utilization of four Cas orthologues, attomolar sensitivity quantification, increasing the sensitivity due to implementation of the Csm6 molecule and lastly, the possibility to provide lateral flow assays. HUDSON (heating extracted diagnostic samples to obliterate nucleases) is the sample preparation technique created for the direct release of viral RNA from natural/raw samples (urine, saliva, whole blood, serum and plasma), so they can be proceeded to the SHERLOCK analysis without complex preparation and purification steps, while maintaining genuine sample consistency [7]. A noteworthy adaptation that provides a faster and simpler assay flow is fusion of the pre-amplification and Cas13-based detection in a one-pot assay that enables result observation within 10-15 minutes at the femtomolar concentration range. Additionally, the one-pot SHERLOCK platform is less susceptible to contamination [11], mainly because there is no need for sample transfer between different spots of the assay.

Applications of the SHERLOCK assay

Nucleic acid detection is one of the main approaches to identify the pathogen that caused a certain condition. The aim to develop new strategies that will provide higher sensitivity and specificity along with low cost and operability in environments with limited resources are the reasons why present researches tend to simplify existing or establish *ne novo* analytic procedures. The SHERLOCK assay is used for detecting several different agents including viruses, bacteria, fungi and even some biomarkers [4]. A couple of recent applications of the abovementioned assay are listed in the Table 1.

Table 1. Application of the SHERLOCK platform for pathogen detection

Sample	Pathogen	Resulting signal	Time (minutes)	Reference
Saliva, urine	EBOV, LASV	Fluorescence Lateral flow	>120	[13] (2020)
Nasopharyngeal and throat swab	SARS-CoV-2	Fluorescence	70	[14] (2020)
DNA sample extracted from *Yersinia* strains	*Yersinia pestis*		240	[15] (2021)
84 clinical isolates Urine	*Neisseria gonorrhoeae*		>300	[16] (2022)
Dried bool spot	*Plasmodium falciparum*		>210	[17] (2021)
Cervical swab	*Chlamydia trachomatis*		120	[18] (2021)
25 clinical isolates 43 BALF	*Aspergillus fumigatus*		70	[19] (2022)
Laboratory strains	CCHFV		30-40	[20] (2022)

BALF - Bronchoalveolar lavage fluid; EBOV – Ebola virus; LASV – Lassa virus; SARS-CoV-2 – Severe acute respiratory syndrome coronavirus 2

Conclusion

The CRISPR/Cas system, naturally responsible for the adaptive immune response in some prokaryotic cells, after determining its abilities, became the main system for genetic rearrangement and manipulation. The utilization of this system is based on the effector cleavage activity with single-base specificity. Combining the CRISPR/Cas system with nucleic acid pre-amplification methods results in a rapid, portable, versatile, cost-effective and highly sensitive method that can be used in point-of-care (POC) testing or for pathogen detection, especially in an environment with limited resources. The SHERLOCK platform serves as a prototype of the previously mentioned union and its full capabilities are yet to be clearly determined. The great potential of this assay relies on high sensitivity and quite resistance to contamination, but the disadvantages such as a strong preference for purified samples and relatively complex pre-analytic sample preparation still need to be conquered.

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SHERLOCK – PLATFORMA ZA OTKRIVANJE NUKLEINSKIH KISELINA

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Sažetak: *Nove analitičke strategije bazirane na savremenim tehnološkim postignućima imaju znatan uticaj na modernu mikrobiološku dijagnostiku. Genetska informacija pohranjena unutar nukleinskih kiselina postala je ciljno „mjesto" mnogih molekularnih dijagnostičkih metoda primjenjenih u cilju otkrivanja i prepoznavanja različitih mikroorganizama. Svjesnost o raznolikosti patogena koji mogu izazvati lokalne i sistemske manifestacije važna je stavka prilikom očuvanja svjetskog zdravlja stoga precizno, brzo i pouzdano razlikovanje uzročnika obezbjeđuje odgovarajuć odgovor. Otkriće i razumijevanje mehanizama funkcionisanja grupisanih uobičajeno isprekidanih kratkih palindromskih ponavljanja (eng. Clustered Regularly Interspaced Short Palindromic Repeats, CRISPR) udruženih sa Cas proteinima uveliko je omogućilo razvoj nekoliko tehnologija koje već jesu ili će biti uvrštene u kliničku primjenu. Dva Cas proteina, Cas12 i Cas13, predstavljaju glavne enzime koji se nastoje kombinovati sa metodama pre-amplifikacije nukleinskih kiselina u cilju postizanja sekvekvencionalno-specifičnog prepoznavanja. Specifični visoko-osjetljivi enzimski reporter (eng. Specific High Sensitivity Enzymatic Reporter UnLOCKing, SHERLOCK) je prototip prethodno navedenog spoja. Multipleksno, ultra-osjetljivo i visoko-specifično otkrivanje RNA i DNA iz pojedinog kliničkog uzorka izvodivo je posredstvom SHERLOCK platforme u kratkom vremenskom intervalu. Daljnje istraživanje predmetne metode praćeno novim implementacijama osiguraće kontinuiran napredak mikrobiološke dijagnostike. Predmetni rad opisuje osnovne principe rada SHERLOCK platforme te naglašava mogućnosti primjene iste u kliničkoj dijagnostici.*

Ključne riječi: *SHERLOCK, nukleinske kiseline, detekcija*